Saskatchewan Agriculture, Food and Rural Revitalization

ADF

AGRICULTURE DEVELOPMENT FUND

FINAL REPORT

20020141

TESTING OF PROTOTYPE VACCINES FOR PASTEURELLA MULTOCIDA PNEUMONIA IN CATTLE

Funded by: The Agriculture Development Fund

March, 2005

Prepared by: Vaccine & Infectious Disease Organization

106-01268/

FINAL REPORT

AGRICULTURE DEVELOPMENT FUND

PROJECT #20020141

"Testing of prototype vaccines for *Pasteurella multocida* pneumonia in cattle"

by

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February 25, 2005

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EXECUTIVE SUMMARY

Pasteurella multocida is one of the three principal bacterial pathogens associated with bovine respiratory disease. Unfortunately, efficacious vaccines for the prevention of infection by this organism have not been available for use in cattle. We have previously characterized several antigens of *M. haemolytica* and *H. somnus*, including two proteins named LppB and TfbA. The former is an outer membrane lipoprotein of unknown function, while the latter is one of a family of bacterial proteins involved in the acquisition of iron from its host. We have demonstrated the vaccine potential of both proteins in other pathogens and have also shown that *P. multocida* contains the genes coding for these proteins and produces similar, but not identical, molecules. Thus, they represent attractive targets for *P. multocida* vaccine development.

We have successfully isolated the genes coding for both of these proteins using PCR amplification of genomic DNA and have expressed them in an *Escherichia coli* expression vector. The *E. coli* strains produce significant quantities of protein and we purified approximately 5 mg of LppB and TfbA for vaccine testing. A dose titration experiment was been carried out to determine the minimal immunizing dose required in cattle, and the results obtained indicate that doses as low as 12.5 micrograms of each antigen induced similar immunological responses to higher doses. However, when the antigens were combined in the same formulation, the response against the TfbA protein was significantly lower, suggesting that antigen interference may be a problem for a combination vaccine. Thus, the proteins were administered in separate vaccine formulations in a trial designed to measure vaccine efficacy. Two groups of ten calves were immunized with a placebo or a combination of the *P. multocida* LppB and TfbA proteins, respectively, and were boosted 21 days later. Ten days following the second immunization, all animals were challenged with Bovine Herpesvirus-1 and *P. multocida*. The results indicate that the experimental vaccine did not provide any degree of protection relative to the placebo and thus we conclude that neither of the LppB or TfbA proteins are protective antigens. These results are discussed in terms of their implication for *P. multocida* vaccine development.

Background

Pasteurella multocida is one of the three principal bacterial pathogens associated with bovine respiratory disease. Unfortunately, efficacious vaccines for the prevention of infection by this organism have not been available for use in cattle. The organism is one of the best characterized bacterial species and was the first veterinary pathogen for which the entire genome was sequenced. We have previously characterized several antigens of *M. haemolytica* and *H. somnus*, including two proteins named LppB and TfbA. The former is an outer membrane lipoprotein of unknown function, while the latter is one of a family of bacterial proteins involved in the acquisition of iron from its host. We have demonstrated the vaccine potential of both proteins in other pathogens and have also shown that *P. multocida* contains the genes coding for these proteins and produces similar, but not identical, molecules. Thus, they represent attractive targets for *P. multocida* vaccine development.

Objectives

The long-term objective of this project is to develop combination vaccines for the prevention of bovine respiratory disease. The research described in this proposal will cover the following specific aims:

1) Isolation and characterization of the genes coding for *P. multocida* LppB and TfbA.
2) Production of large quantities of protein for vaccine trials.
3) Determination of the serological response to immunization with the recombinant LppB and TfbA proteins.
4) Testing of their protective capacity in an experimental disease model.

Project Results

1. Isolation of genes coding for *P. multocida* LppB and TfbA: We had previously determined that bovine strains of *P. multocida* produced both the LppB and TfbA proteins. Thus, the genes coding for these proteins were isolated by PCR using primers based upon the sequence of homologous genes from other members of the Pasteurellaceae. The sequences of the primers are described in Table 1. Following PCR, the genes were cloned into an expression vector (pGH433) and the level of protein expression was monitored by polyacrylamide gel electorphoresis and Western blotting. Figure 1 shows the proteins produced by the recombinant strains.

Gene	Primer	Sequence
TfbA	Forward	5'-gccggatcccttactttagtctttagtgtg-3'
	Reverse	5'-cgcggatccctaaaaacgaatttccaccga-3'
LppB	Forward	5'-cggagatctaaaaagtcgttttactg-3'
	Reverse	5'-ataggatcctccgatgcagttcttcac-3'

Table 1: Nucleotide sequences of primers used for PCR amplification

The genes coding for each antigen were sequenced using conventional techniques and the full nucleotide sequence of each gene is shown in Figures 1 and 2. Both genes show a high degree of homology to homologues found in *Mannheimia haemolytica*, *Haemophilus somnus* and other bacteria yet are different enough to suggest that cross protection between these species would not occur following either immunization or natural infection.

2. Antigen Production: In order to complete the two vaccine studies proposed for this project, approximately 20 mg of each protein would be required. Thus, antigen production experiments were carried out at the 1-2 L scale, using a combination of conventional ion-exchange chromatography and differential centrifugation to purify each protein. The plasmid expression vector used to express each gene contained an inducible *tac* promoter element and therefore the production of protein could be induced in bacterial cultures by the addition of isopropyl-β,D-thiogalactoside (IPTG), a lactose analog. Therefore, cultures were grown to the

mid-logarithmic phase of growth at which time IPTG was added to a final concentration of 1 mM. After a further 4 hours incubation at 37°C, cells were harvested by centrifugation and lysed by sequential treatment with lysozyme and Triton X-100 detergent. The TfbB protein was expressed in the form of insoluble inclusion bodies (Figure 3) which were easily purified by differential centrifugation. LppB was produced as a mixture of soluble and insoluble protein (Figure 3), but the latter was not in the form of typical inclusion bodies. Thus, the insoluble material was removed by centrifugation and the soluble protein was further purified by conventional ion-exchange chromatography. The yield of each component was approximately 10 mg of purified protein per litre of culture, an amount consistent with our past experience with these antigens from other bacterial species. The LppB protein was easily purified to approximately 95% purity (see Figure 4) as judged by polyacrylamide gel electrophoresis, while the TfbA protein was only approximately 70% pure. The purification of the antigens is shown in Figures 3 and 4, with the former representing a pilot scale study and the latter showing the final protein purity used in the two vaccine trials described below.

```
ID   PMTBPAF001 PRELIMINARY; DNA; 2650 BP.
DE   MELD CREATED BY ASSEMGEL REV 2.6
SQ   SEQUENCE  2650 BP;  799 A;  484 C;  574 G;  793 T;
     GTCCGGTAAC GTGCTGAGCC CGGCCAAGCT TACTCCCCAT CCCCCTGTTG ACAATTAATC
     ATCGGCTCGT ATAATGTGTG GAATTGTGAG CGGATAACAA TTTCACACAG GAAACAGGAT
     CACTAAGGAG GTTTAAATAT GGCTACTGTT AACAGATCTA AGCTTCCCGG GCTTACTTTA
     GTCTTTAGTG TGATAAGGAG ATCATTTATG AAATTTAAAT ATTGCGTTAT TCATCAATTA
     GTTCTTTCTG GCAGTTTGAT ATTTCCTACA TTAAGTTTTG CAGATTCGGA TTCAACAAAA
     ACAACGCCAA ATAAAGCTAG CTATTTGGAG GAAATTTCCG TTGTCGCAAC GACGGTTCAA
     CATATTTTTT CCTCAACGCA ATCTACCGAA CAATTGCGTA AAAATATGGT GTCTAATGAA
     CGGGATTTGT TTCGCTATGA GGTAGGGATT GGTGTACCAG AATCCGGGAG ATCCGGCAGT
     AATGGCTTTG CTATCCGTGG CGTTGATAAA GATCGTGTCG CTGTTATTGT TGATGGAATA
     CCGCAGGCTG AATCGACTAT ATCTACTTCC GCACGTTATT CGACTGAACG TCATAATGGT
     AATATTAATA ATATTGAATA CGAAAATGTT AGTTCGTTGA AAGTTCAAAA AGGGGCAGCT
     TCTGTAATGT ATGGTAGCGG TGCGTTAGGT GGAACCGTCC AGTTTACCAC AAAAGATATT
     GAGGACTTTG TCGAACCTGG TCGCCATTTG GGCTTTTTGT CTAAAACCGG CTATACTTCA
     AAAAACAGAG AATATCGTCA AGTCATCGGA GTTGGAGGGA AAGGGGAACA CTTTTTTGGT
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     ATTCTCGGCG AACATCGAGG TAAACCCAAT CCGCTCAAGT ACTATACTAC ATCATGGTTA
     ACGAAAGTCG GTTACGATAT TAATAACACT CATCGTTTTA CACTGTTTTT AGAAGATCGC
     CGTGAAAAGA AGCTTACCGA AGAAAAAACA TTAGGGCTTA GTGATGCAGT GCGTTTTGCT
     AATGATCAAA CCCCTTATCT CCGTTATGGT ATTGAATATC GATATAACGG CTTGTCTTGG
     TTGGAAACGG TAAAGCTTTT TTTGGCAAAG CAGAAAATCG AACAACGTTC TGCTCTCCAA
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     CGTTATCATA ACTTTATTCA AGAACGTGAG ATGACCTGTG ATAAAATTCC ATATGAGTAT
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     AGGGGGTATA CTTCCCGTTC TGTCCGTGCT AACAGCCCAG GCATTAATCG GTTTACCGCA
     CCAAAACGTA ATTTTGCTGC CTCGGTGGAA ATTCGTTTTT AGGGATCCTA GCTAGCTAGC
     CATGGCATCA CAGTATCGTG ATGACAGAGG CAGGGAGTGG GACAAAATTG AAATCAAATA
     ATGGATTTTA TTTTGACTGA TAGTGACCTG TTCGTTGCAA CAAATTGATA AGCAATGCTT
     TTTTATAATG
```

Figure 1: Nucleotide sequence of *P. multocida* TfbA. The ATG and TAG codons in bold type represent the start and end of the gene, respectively.

```
ID   PMLPPBF001      PRELIMINARY;   DNA;  1444 BP.
DE   MELD CREATED BY ASSEMGEL REV 2.0
SQ   SEQUENCE   1444 BP;   426 A;   320 C;   323 G;   373 T; 2 OTHER;
     CNNGCATCAC TAAGGAGGTT TAAATATGGC TACTGTTAAC AGATCTAAAA AGTCGTTTTT
     ACTCTTGCCA ATCAGTGTTG CCGTATTGGC TGCCTGTAGT TCAAATACGC CTGCGCGGGT
     AGAGAGTGCA GATGGCAGTT TATCGCCGGG GATGATGCAA CCCGTTGATG CATCAAGTGG
     TGGCACATGG GAGCCACAAA TTCAACAACA AAATACCATG CCAGCGGGAA TGGATCAGTC
     CGTCTATTCT CCTTCTACCT CGCAACCAAG TTTGCCAGTA AGCCATCAAC CAACACCGCC
     GACATCATCT TCTTTCGATA TTCCGCGTAA ACCGGTCACC GGCGAACCAG ATTACAGCAA
     AATTGCCAGA GGCTCTTATC AAGGTGAAAG CTATATGGTG CGTAAGGGCG ACAGTATGTA
     TTTGATTTCT TACATTTCCG GTTTAAGTAT CAAAGAGATC GCTGCGTTAA ATAATCTATC
     AGAGCCTTAT ACTTTAGCGA CAGGACAAGT GTTAAAACTG TCGAATAAAG TGTCAACAAC
     CTCTACCATG TCACCTTCAA CCGCGGTAGG AGAGGTAGGT ACAAATGTAA AGCCGACAAC
     ACAACATTTT GAGATCCCGC GTAATCCAGC GGATAACCGC CCGGATTATA GCAAGATCGA
     TAAAGGTTTT TATAAAGGGG AAACTTATAC GGTGCGTAAA GGCGATACAA TGTACCTCAT
     CGCCTATATT TCAGGCTTAG ATGTCAAAGA ATTGGCATCA TTAAACAATA TGTCTGAACC
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     TGTCAGTTCC GCCCCTGTTC CAGTCCAACC TGAACCAGTG GTTAAGCCTG TTGAAAGCAC
     GTCTGTACCT GTGCCAAGCA CGAGCAGTAA ACACATGGTT TCGAATGTAA CTTGGCAATG
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     TGATATTGCT GGTTCACGTG GTCAAGCGGT GAATGCTGCC GCCGCAGGCA GAGTGGTTTA
     TGCGGGTAAT GCATTACGTG GTTATGGCAA TTTAATTATC ATTAAACACA ATGATGACTA
     TTTAAGTGCC TATGCTCACA ATGAAAGTAT TTTAGTTAAA GATCAGCAAG AAGTTCGTGC
     GGGTCAACAG ATTGCCAAAA TGGGTAGCTC TGGTACCAAT AGTGTCAAAC TTCACTTTGA
     AATTCGCTAT AAAGGCAAAT CAGTTGATCC AACCCGTTAT TTGCCAAAAC GTTAATTCTC
     TGAC
```

Figure 2: Nucleotide sequence of *P. multocida* LppB. The ATG and TAA codons in bold type represent the start and end of the gene, respectively.

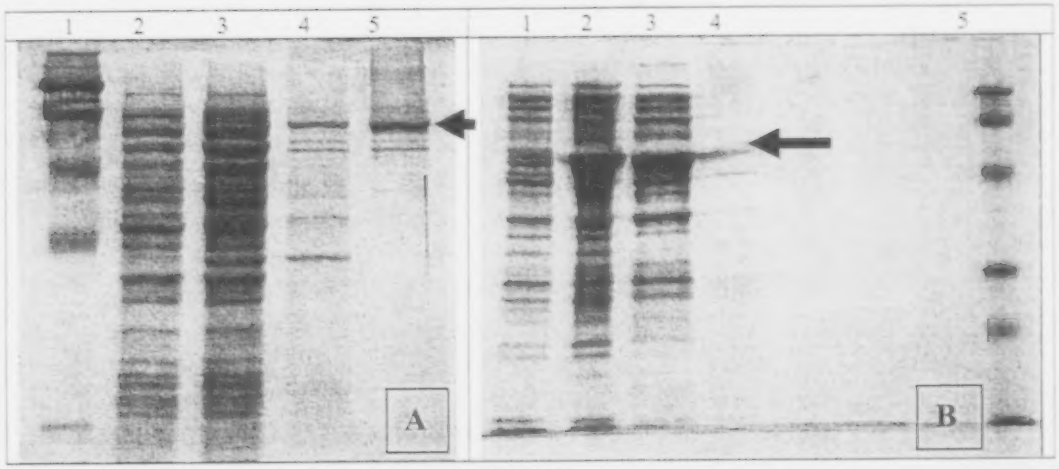

Figure 3: Polyacrylamide gel electrophoresis of samples enriched for *Pasteurella multocida* TfbA and LppB.

Panel A: Lane 1, molecular weight markers; lane 2, whole cells expressing TfbA (uninduced); lane 3, whole cells expressing TfbA (induced with IPTG); lane 4, TfbA insoluble inclusion bodies; lane 5, purified TfbA. Panel B: Lane 1, cells expressing LppB (uninduced); lane 2, cells expressing LppB (induced with IPTG); lane 3, soluble fraction with inclusion bodies removed; lane 4, insoluble LppB; lane 5, molecular weight markers. Arrows show the position of the TfbA and LppB bands.

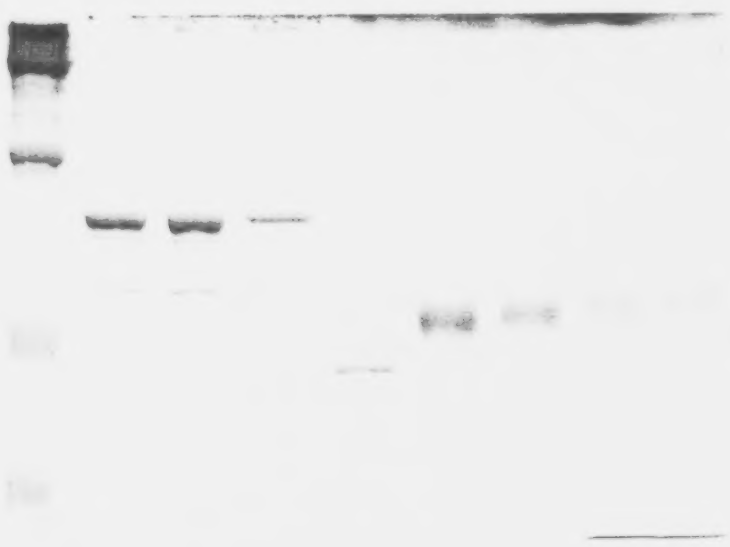

Figure 4: Polyacrylamide gel electrophoresis of recombinant TfbA and LppB.

Lane 1: Molecular weight markers; lanes 2-4, TfbA; lane 5, LppB; lanes 6-9, bovine serum albumin standards.

3. **Serological response to immunization with recombinant LppB and TfbA:** In order to test the minimal immunizing dose of each antigen required in a vaccine, we carried out a vaccine trial to test the immunogenicity of each antigen in cattle at three different doses. The individual proteins were formulated with the adjuvant VSA3 such that each 2 cc dose contained 100 µg, 25 µg or 12 µg of protein. In addition, a vaccine formulation containing both antigens together at a 50 µg dose was included as well as a placebo vaccine which did not contain any *P. multocida* components. The combination vaccine group was included to determine whether or not the presence of both of these components could interfere with the immune response against the other. Groups of ten calves were immunized subcutaneously with these vaccine formulations and were boosted three weeks later. Serum samples were taken from each animal at the time of immunization (days 0 and 21) as well as days 35 and 49.

The immunological response against each antigen was determined using an enzyme-linked immunosorbent assay previously developed for use with homologous antigens from other bacterial species. The results are summarized in Figures 5 and 6 below. Each formulation induced statistically similar immune responses ($p < 0.05$) when the antigens were given separately at the three doses described above. The responses against the LppB protein were higher than those against TfbA, a result expected from previous studies with the equivalent *H. somnus* vaccine studies. No immunological interference between the two antigens was observed when the anti-LppB responses were analyzed. However, the combination vaccine induced significantly lower anti-TfbA antibody titres indicating that the presence of LppB compromised the ability of the animals to respond to TfbA or that the combination of the two proteins altered the conformation of TfbA such that the dominant epitopes were masked. Since the individual proteins induced excellent responses, it was decided to formulate them separately for the final vaccine efficacy experiment (below).

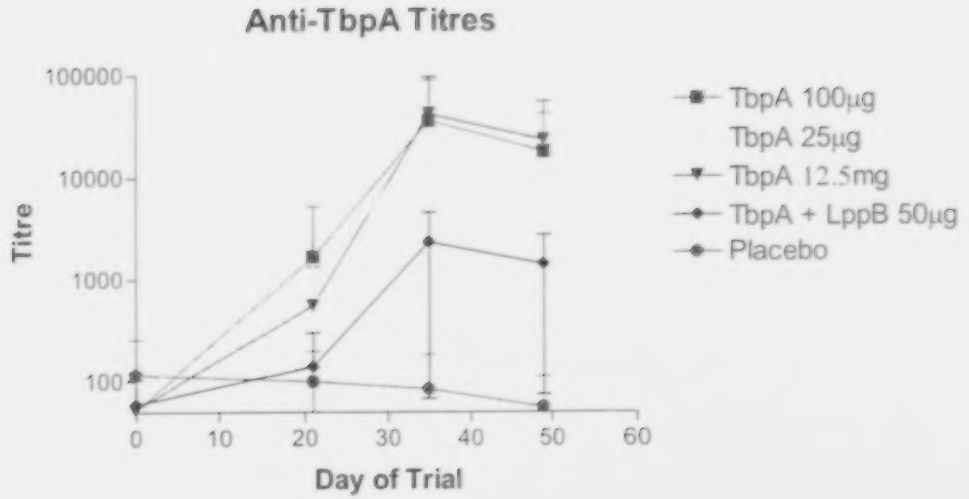

Figure 5: Serological response against the *P. multocida* transferrin-binding protein TfbA.

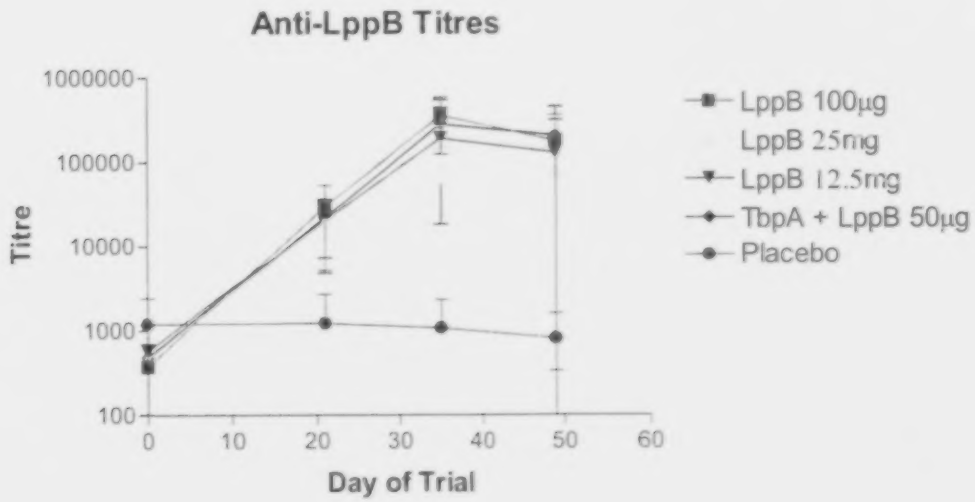

Figure 6: Serological response against the *P. multocida* outer membrane lipoprotein LppB.

4. **Protective capacity of LppB and TfbA:** The data described above indicated that calves immunized with the LppB and TfbA antigens of *P. multocida* responded well in terms of their humoral immune responses. However, this does not predict the level of protection against disease since neither of these antigens has been shown to be protective components of this organism. Therefore, we conducted a second vaccine trial in which groups of 10 8-month old calves were immunized twice as described above with either a placebo formulation containing the adjuvant VSA3 but no antigen, or vaccines containing 50 μg LppB and TfbA formulated in VSA3. For the latter group, we formulated each antigen separately due to the immunological interference which was observed in the dose titration trial described above (Figures 5 and 6). All animals were screened prior to the trial to ensure that they had no background immune response against both bovine herpesvirus-1 and *P. multocida*. Fourteen days following the second immunization, all animals were challenged with virulent bovine herpesvirus-1, followed 4 days later by *P. multocida*. Both virus and bacteria were delivered by aerosol using a nebulizer, a system which has worked well in the past for various bovine respiratory disease models, including *P. multocida*. All animals were monitored daily for clinical signs of disease, including respiratory distress, depression and overall sickness. In addition, both weights and rectal temperatures were recorded daily. This model typically results in approximately 25% mortality in susceptible animals with clinical signs of disease persisting for up to 10 days post-infection. Serum samples were taken from each animal at the time of each immunization and on the day of BHV-1 infection for measurement of immune responses.

The serological response to immunization is illustrated in Figures 12 and 13, and the results indicated that all animals responded well to vaccination. The absolute values were lower for the anti-TfbA responses, a phenomenon which has been observed previously (see above). The LppB protein is lipid-modified at its amino terminus and thus has intrinsic adjuvant activity which is one reason why it makes an excellent vaccine antigen.

The animals responded well to the BHV-1 challenge (Figure 7), with both groups exhibiting elevated temperatures within 48 hours of exposure to the virus. In the absence of a secondary bacterial infection, temperatures usually return to normal with four days as is illustrated in

Figure 7. In this experiment, temperatures remained elevated following exposure to *P. multocida*, indicating a successful bacterial infection. However, no differences were observed between vaccinated and control animals, and in all cases temperatures returned to normal by the end of the trial. This trend was similar for weight loss, with no significant differences observed between vaccine groups (Figure 8).

Despite the success of the bacterial challenge, clinical scores were very low (Figures 9-11), with only five animals in each group showing any clinical signs of disease. One vaccinated animal was euthanized on the last day of the trial, but a postmortem analysis showed the presence of both *P. multocida* and *Haemophilus somnus*. This challenge model typically results in approximately 25% mortality, and therefore the results of this study would suggest that all animals were more resistant than those used in the past. While we routinely screen sera from animals for the presence of antibodies against both *P. multocida* polysaccharide as well as antibodies to the specific vaccine antigens (LppB and TfbA), and all animals selected for the trial were considered negative. However, we cannot rule out the possibility that they had been exposed to another serotype of *P. multocida* as the ELISA procedure used employs capsular polysaccharide as a diagnostic antigen. We are currently determining if this was the case by measuring the pre-vaccination antibody levels to other capsular serotypes. Regardless of the reason for the lack of mortality, one would have expected the prototype vaccine to have shown an effect in the face of a mild challenge, and we did not detect any differences between the two groups. Thus, we conclude that unlike other members of the Pasteurellaceae, the LppB and TfbA proteins of *P. multocida* are not protective antigens and are thus of no value for a respiratory disease vaccine. There does remain a possibility that they may be protective in a hemorrhagic septicemia disease model, since others have shown the TfbA appears to be a target of the immune response in infected buffalo (Mukkar, T., personal communication). However, this is clearly not relevant to the situation in Saskatchewan, or any other location outside of Africa and Asia.

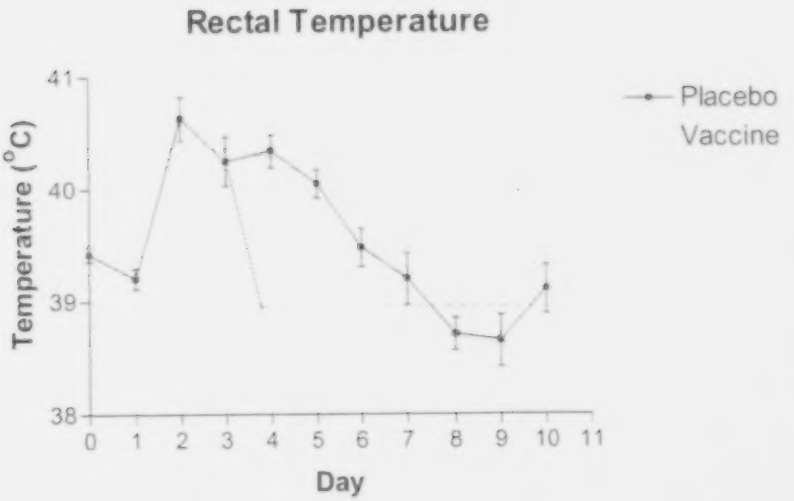

Figure 7: Rectal temperatures in challenged animals. The dashed line represents expected temperatures in the absence of a secondary bacterial challenge. Error bars indicate standard deviation values.

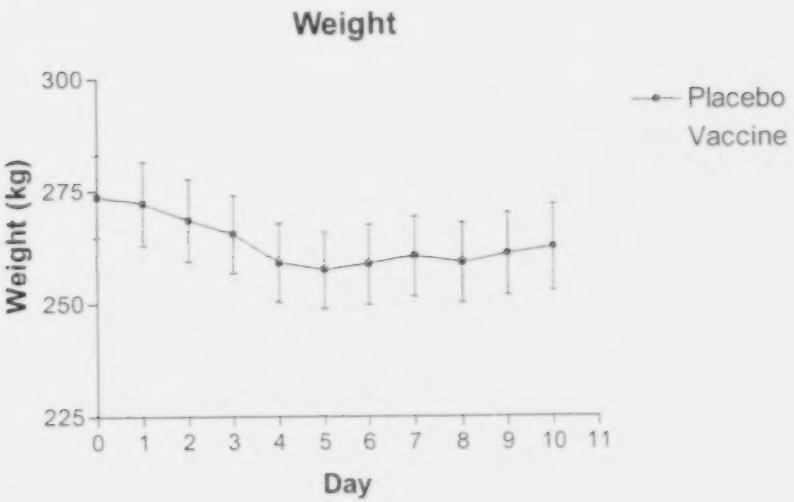

Figure 8: Mean daily weights in challenged animals. Error bars indicate standard deviations.

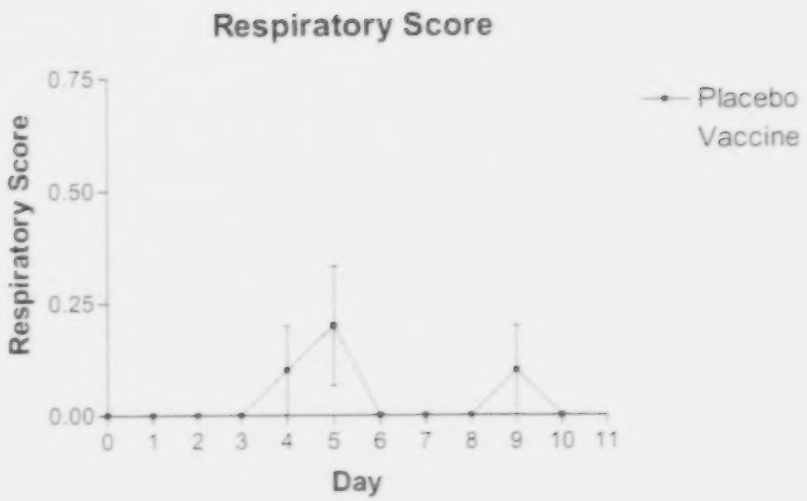

Figure 9: Mean respiratory scores following challenge.

Figure 10: Mean depression scores following challenge.

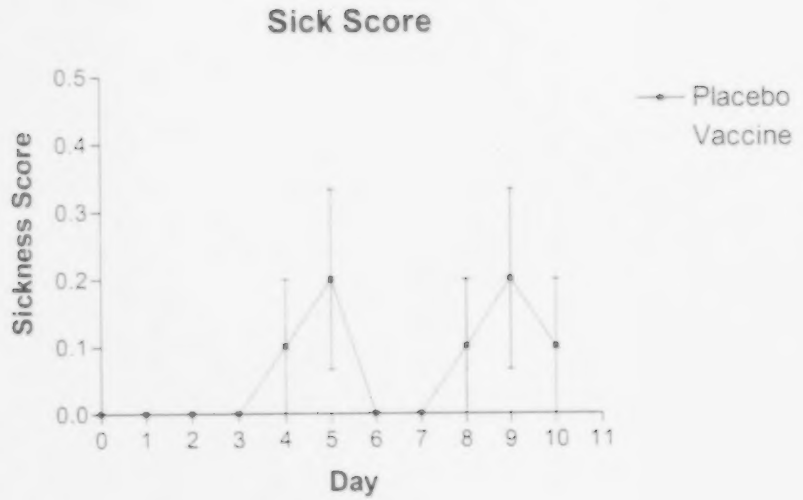

Figure 11: Mean sick scores following challenge.

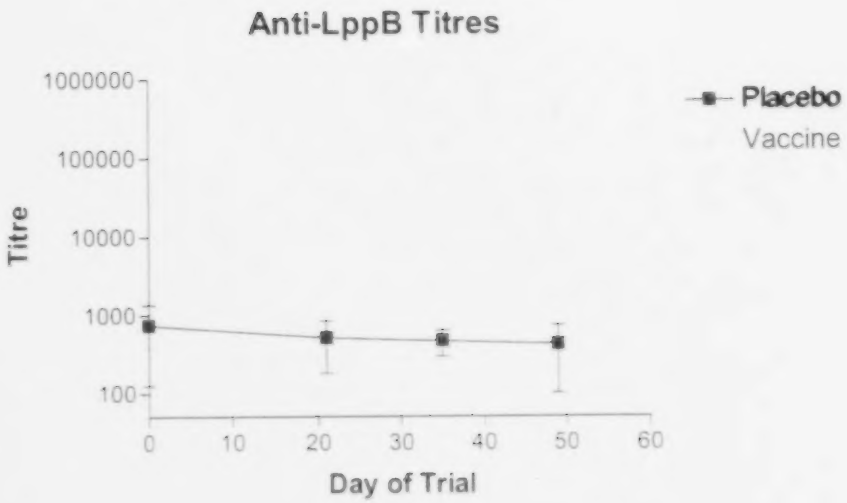

Figure 12: Serological response to LppB following immunization.

The response to immunization was measured using an enzyme-linked immunosorbent assay. The anti-LppB titres are expressed as the reciprocal of the highest dilution which was greater than the background plus two standard deviations. Values are presented as means and the vaccinated group had significantly higher titres on all days with the exception of the pre-immunization sample ($p<.05$)

Figure 13: Serological response to TfbA following immunization.

The response to immunization was measured using an enzyme-linked immunosorbent assay. The anti-TfbA titres are expressed as the reciprocal of the highest dilution which was greater than the background plus two standard deviations. Values are presented as means and the vaccinated group had significantly higher titres on all days with the exception of the pre-immunization sample ($p<.05$)

PROJECT-DEVELOPED MATERIALS

Not applicable.

PERSONNEL

Salary support has been used for Ms. Tracy Prysliak, who has spent 50% of her time on project activities. A total of $11,369 has been used for salary support.

EQUIPMENT

No equipment was purchased as part of this project.

ACKNOWLEDGEMENT

At the present time, no publications or presentations based on this project have been submitted. We will ensure that Saskatchewan ADF is acknowledged when a formal publication is prepared.

ICAR DATA ENTRY

ICAR Project #33331317.